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(54) Title: INHIBITION OF CELL DIVISION BASED USE OF ANALOGS OF NEUROPEPTIDE Y

(57) Abstract: We describe peptides with cell-cycle inhibitory and anti-angiogenic activity and their use in the treatment of diseases which would benefit from the inhibition of cell division and/or angiogenesis, for example cancer.

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INHIBITION OF CELL DIVISION BASED USE OF ANALOGS OF NEUROPEPTIDE Y

The invention relates to peptides with cell-cycle inhibitory and anti-angiogenic activity and their use in the treatment of diseases which would benefit from the inhibition of cell division and/or angiogenesis, for example cancer.

The eukaryotic cell cycle is divided into distinct phases, G1 (cell growth and preparation for DNA synthesis), S (DNA synthesis) G2 (preparation for mitosis), M (mitosis, nuclear division). The cell-cycle is highly regulated and many diseases are characterised by abnormal cell-division, for example, cancer or psoriasis.

Angiogenesis, the development of new blood vessels from an existing vascular bed, is a complex multistep process that involves the degradation of components of the extracellular matrix and then the migration, cell-division and differentiation of endothelial cells to form tubules and eventually new vessels. Angiogenesis is involved in pathological conditions such as tumour cell growth; non-cancerous conditions such as neovascular glaucoma; inflammation; diabetic nephropathy; retinopathy; rheumatoid arthritis; inflammatory bowel diseases (eg Crohn's disease, ulcerative colitis); and psoriasis.

The vascular endothelium is normally quiescent. However upon activation endothelial cells proliferate and migrate to form microtubules which will ultimately form a capillary bed to supply blood to developing tissues and, of course, a growing tumour. A number of growth factors have been identified which promote/activate endothelial cells to undergo angiogenesis. These include, by example and not by way of limitation; vascular endothelial growth factor (VEGF); transforming growth factor (TGF β); acidic and basic fibroblast growth factor (aFGF and bFGF); and platelet derived growth factor (PDGF), Folkman, Nature Medicine, 1: 27-31, 1995; Leek et al J. Leuk. Biol., 56: 423-35, 1994.

A further disease characterised by uncontrolled cell division is psoriasis. Psoriasis is a generic term to cover a range of diseases characterized by abnormal proliferation of skin cells. The disease covers the following list which is not exhaustive but merely illustrative: nail psoriasis; scalp psoriasis; plaque psoriasis; pustular psoriasis; guttate psoriasis; inverse psoriasis; erythrodermic psoriasis; psoriatic arthritis.

Psoriasis is one of the most frequent skin diseases, affecting 1-3% of the Caucasian population world wide. The disease is characterised by alterations in a variety of different cell types. These include epidermal keratinocytes which are characterised by hyperproliferation and an altered differentiation which is indicated by focal parakeratosis and aberrant expression of keratinocyte genes encoding hyperproliferation-associated keratin pair 6/16, involucrin, fillagrin, and integrin adhesion molecules (eg VLA-3, 5, 6).

According to an aspect of the invention there is provided a peptide comprising the amino acid sequence ARYYSALRHYINLITRQRT, or a variant peptide wherein said sequence is modified by addition, deletion or substitution of at least one amino acid residue, for use as a pharmaceutical agent.

In a preferred embodiment of the invention there is provided a pharmaceutical composition comprising a peptide according to the invention.

According to an aspect of the invention there is provided a peptide comprising an amino acid sequence, ARYYSALRHYINLITRQRT, or part thereof, or variant thereof, which has been modified by addition, deletion or substitution of at least one amino acid residue, wherein said peptide has cell-cycle inhibitory activity, for the manufacture of a medicament for use in the treatment of diseases or conditions which would benefit from the inhibition of cell division.

According to a further aspect of the invention there is provided an isolated nucleic acid molecule which encodes a peptide characterised in that the nucleic acid molecule is selected from the following group:

- 5 i) a nucleic acid molecule comprising the nucleic acid sequence presented in figure 6;
- ii) a nucleic acid molecule as represented by the sequence presented in figure 6 which has been modified by addition, deletion or substitution of at least one nucleotide base within at least one codon to encode a variant peptide which has cell-cycle inhibitory activity;
- 10 iii) a nucleic acid molecule which hybridizes to the sequence in (i) or (ii); and
- iv) a nucleic acid molecule comprising a nucleic acid sequence which is degenerate as a result of the genetic code to the sequences identified in (i)-(iii); for the manufacture of a medicament for use in the treatment of diseases or conditions which would benefit from an inhibition of cell-division.

15

According to a further aspect of the invention there is provided a peptide encoded by the nucleic acid according to the invention for the manufacture of a medicament for use in the treatment of diseases or conditions which would benefit from the inhibition of cell division.

20

In a further preferred embodiment of the invention said peptide inhibits angiogenesis.

In a preferred embodiment of the invention said disease is selected from the group consisting of: cancer; psoriasis; neovascular glaucoma; rheumatoid arthritis; diabetic
25 retinopathy.

In a further preferred embodiment of the invention said disease is cancer.

In a yet further preferred embodiment of the invention said disease is psoriasis.

30

Preferably said psoriatic condition is selected from the group consisting of: nail psoriasis; scalp psoriasis; plaque psoriasis; pustular psoriasis; guttate psoriasis; inverse psoriasis; erythrodermic psoriasis; psoriatic arthritis.

- 5 Reference to cell-cycle inhibition and anti-angiogenic activity is determined by assays hereindisclosed. For example, the peptides of the invention are tested by *in vitro* assays which include the inhibition of endothelial cell mediated tubule formation, inhibition of endothelial cell migration, inhibition of VEGF and bFGF induced endothelial cell proliferation and endothelial cell cytotoxicity assays, FACS
10 analysis of cell cultures which have been exposed to peptides of the invention. Peptides can also be tested in vivo using murine tumour models as hereindisclosed.

A variant , (i.e. a fragment peptide and reference peptide) may differ in amino acid sequence by one or more substitutions, additions, deletions, truncations which may
15 be present in any combination. Among preferred variants are those that vary from a reference polypeptide by conservative amino acid substitutions. Such substitutions are those that substitute a given amino acid by another amino acid of like characteristics. The following non-limiting list of amino acids are considered conservative replacements (similar): a) alanine, serine, and threonine; b) glutamic
20 acid and asparatic acid; c) asparagine and glutamine d) arginine and lysine; e) isoleucine, leucine, methionine and valine and f) phenylalanine, tyrosine and tryptophan. Most highly preferred are variants which retain the same biological function and activity as the reference peptide from which it varies.

- 25 A peptide according to the invention is a variant wherein one in which one or more amino acid residues are substituted with conserved or non-conserved amino acid residues, or one in which one or more amino acid residues includes a substituent group. Conservative substitutions are the replacements, one for another, among the aliphatic amino acids Ala, Val, Leu and Ile; interchange of the hydroxyl residues Ser
30 and Thr; exchange of the acidic residues Asp and Glu; substitution between amide

residues Asn and Gln; exchange of the basic residues Lys and Arg; and replacements among aromatic residues Phe and Tyr.

In addition, the invention features peptide sequences having at least 75% identity with the peptide sequences as hereindisclosed, or fragments and functionally equivalent peptides thereof. In one embodiment, the peptides have at least 85% identity, more preferably at least 90% identity, even more preferably at least 95% identity, still more preferably at least 97% identity, and most preferably at least 99% identity with the amino acid sequences illustrated herein.

10

In a preferred embodiment of the invention said peptide comprises an amino acid sequence, or part thereof, consisting of the sequence ARYYSALRHYINLITRQRT. Preferably said peptide is a peptide consisting of the sequence ARYYSALRHYINLITRQRT.

In a further preferred embodiment of the invention said peptide is a fragment ARYYSALRHYINLITRQRT. Preferably said fragment is at least 3 amino acid residues in length, 4 amino acid residues in length, 5 amino acid residues in length or 6 amino acid residues in length, 7 amino acids in length, 8 amino acids in length, 9 amino acids in length, 10, amino acids in length, 11 amino acids in length, 12 amino acids in length, 13 amino acids in length, 14 amino acids in length, 15 amino acids in length, 16 amino acids in length, 17 amino acids in length, or 18 amino acids in length.

In a yet further preferred embodiment of the invention said peptide is at least 20 amino acid residues in length; 21 amino acid residues; 22 amino acid residues; 23 amino acid residues; 24 amino acid residues; 25 amino acid residues; 26 amino acid

residues; 27 amino acid residues; 28 amino acid residues; 29 amino acid residues; 30 amino acid residues in length.

5 In a further preferred embodiment of the invention said peptide is at least 35 amino acid residues in length; at least 40 amino acid residues; at least 45 amino acid residues; at least 50 amino acid residues; at least 60 amino acid residues; at least 70 amino acid residues; at least 80 amino acid residues; at least 90 amino acid residues; at least 100 amino acid residues in length.

10 It will be apparent to one skilled in the art that modification to the amino acid sequence of peptides according to the invention could enhance the binding and/or stability of the peptide with respect to its target sequence. In addition, modification of the peptide may also increase the in vivo stability of the peptide thereby reducing the effective amount of peptide necessary to inhibit cell-division or angiogenesis or
15 to induce apoptosis. This would advantageously reduce undesirable side effects which may result in vivo. Modifications include, by example and not by way of limitation, acetylation and amidation.

In a preferred embodiment of the invention said peptide sequence is acetylated.
20 Preferably said acetylation is to the amino terminus of said peptide.

In a further preferred embodiment of the invention said peptide sequence is amidated. Preferably said amidation is to the carboxyl-terminus of said peptide.

25 In a further preferred embodiment of the invention said peptide, or fragment thereof, is modified by both acetylation and amidation.

It will be apparent to one skilled in the art that fragments of a peptide as herein disclosed, are susceptible to modifications such as acetylation and/or amidation.

30

Alternatively or preferably, said modification includes the use of modified amino acids in the production of recombinant or synthetic forms of peptides according to the invention. It will be apparent to one skilled in the art that modified amino acids include, by way of example and not by way of limitation, 4-hydroxyproline, 5-hydroxylysine, N⁶-acetyllysine, N⁶-methyllysine, N⁶,N⁶-dimethyllysine, N⁶,N⁶,N⁶-trimethyllysine, cyclohexylalanine, D-amino acids, ornithine. Other modifications include amino acids with a C₂, C₃ or C₄ alkyl R group optionally substituted by 1, 2 or 3 substituents selected from halo (eg F, Br, I), hydroxy or C₁-C₄ alkoxy.

Alternatively, fragments could be synthesised de novo and also modified by, for example, cyclisation. Cyclisation is known in the art, (see Scott et al Chem Biol (2001), 8:801-815; Gellerman et al J. Peptide Res (2001), 57: 277-291; Dutta et al J. Peptide Res (2000), 8: 398-412; Ngoka and Gross J Amer Soc Mass Spec (1999), 10:360-363.

In a preferred embodiment of the invention the peptides according to the invention are modified by cyclisation.

According to a further aspect of the invention there is provided an agent comprising two or more peptides according to the invention wherein said agent has cell-cycle inhibitory activity.

In a preferred embodiment of the invention said two or more peptides are linked by a linker molecule. Preferably said linker molecule is a flexible linker.

In a further preferred embodiment of the invention said agent comprises a plurality of peptides according to the invention. Preferably said agent has 3, 4, 5, 6, 7, 8, 9, or 10 peptides linked together as an oligomeric peptides. Preferably said peptide has greater than 10 peptides according to the invention.

In, a further preferred embodiment of the invention said agent is a dimer of two peptides according to the invention.

5 In a further preferred embodiment of the invention said linker is a peptide linking molecule. In a preferred embodiment of the invention said peptide linking molecule comprises at least one amino acid residue which links at least two peptides according to the invention.

10 In a further preferred embodiment of the invention said peptide linking molecule comprises at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acid residues. In a further embodiment of the invention said linking molecule comprises more than 10 amino acid residues.

15 In an alternative embodiment of the invention, the agent is a fusion protein comprising an inframe translational fusion of the peptides according to the invention.

20 It will be apparent that the invention encompasses the formation oligomeric polypeptides which comprise identical peptides according to the invention, herein referred to as homo-oligomeric agents as well as polypeptides comprising different modified peptides, referred to as hetero-oligomeric agents.

25 It will be apparent to one skilled in the art that alternative linkers can be used to link peptides, for example the use of chemical protein crosslinkers. For example homo-bifunctional crosslinker such as disuccinimidyl-suberimidate-dihydrochloride; dimethyl-adipimidate-dihydrochloride; 1,5,-2,4 dinitrobenzene or hetero-bifunctional crosslinkers such as N-hydroxysuccinimidyl 2, 3-dibromopropionate; 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride; succinimidyl 4-[n-maleimidomethyl]-cyclohexane-1-carboxylate.

30 According to a further aspect of the invention there is provide the use of an agent according to the invention as a pharmaceutical.

According to a yet further aspect of the invention there is provided a pharmaceutical composition comprising an oligomeric agent according to the invention.

- 5 When administered, the pharmaceutical compositions of the present invention are administered in pharmaceutically acceptable preparations. Such preparations may routinely contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers, supplementary immune potentiating agents such as adjuvants and cytokines and optionally other therapeutic agents, such as
10 chemotherapeutic agents.

The pharmaceutical compositions of the invention can be administered by any conventional route, including injection or by gradual infusion over time. The administration may, for example, be oral, intravenous, intraperitoneal, intramuscular,
15 intracavity, subcutaneous, or transdermal.

The compositions of the invention are administered in effective amounts. An "effective amount" is that amount of a composition that alone, or together with further doses, produces the desired response. In the case of treating a particular
20 disease, such as cancer, the desired response is inhibiting the progression of the disease. This may involve slowing the progression of the disease temporarily, although more preferably, it involves halting the progression of the disease permanently. This can be monitored by routine methods.

25 Such amounts will depend, of course, on the particular condition being treated, the severity of the condition, the individual patient parameters including age, physical condition, size and weight, the duration of the treatment, the nature of concurrent therapy (if any), the specific route of administration and like factors within the knowledge and expertise of the health practitioner. These factors are well known to
30 those of ordinary skill in the art and can be addressed with no more than routine experimentation.

The pharmaceutical compositions used in the foregoing methods of treatment preferably are sterile and contain an effective amount of peptide, oligomeric agent or nucleic acid encoding said peptide or oligomeric agent, for producing the desired response in a unit of weight or volume suitable for administration to a patient.

The doses of peptide, oligomer, or nucleic acid encoding said peptide/oligomer administered to a subject can be chosen in accordance with different parameters, in particular in accordance with the mode of administration used and the state of the subject. Other factors include the desired period of treatment. In the event that a response in a subject is insufficient at the initial doses applied, higher doses (or effectively higher doses by a different, more localized delivery route) may be employed to the extent that patient tolerance permits.

When administered, the therapeutic preparations of the invention are applied in therapeutically-acceptable amounts and in pharmaceutically-acceptable compositions. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. Such preparations may routinely contain salts, buffering agents, preservatives, compatible carriers, and optionally other therapeutic agents. When used in medicine, the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically-acceptable salts thereof and are not excluded from the scope of the invention.

Peptide/oligomer polypeptide compositions may be combined, if desired, with a pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier" as used herein means one or more compatible solid or liquid fillers, diluents or encapsulating substances which are suitable for administration into a human. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application.

The pharmaceutical compositions may contain suitable buffering agents, including: acetic acid in a salt; citric acid in a salt; boric acid in a salt; and phosphoric acid in a salt.

- 5 The pharmaceutical compositions also may contain, optionally, suitable preservatives, such as: benzalkonium chloride; chlorobutanol; parabens and thimerosal.

10 The pharmaceutical compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy. All methods include the step of bringing the active agent into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the active compound into association with a liquid carrier, a finely divided solid carrier, or both, and then, if
15 necessary, shaping the product.

Compositions suitable for oral administration may be presented as discrete units, such as capsules, tablets, lozenges, each containing a predetermined amount of the active compound. Other compositions include suspensions in aqueous liquids or
20 non-aqueous liquids such as a syrup, elixir or an emulsion.

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous or non-aqueous preparation of peptides/oligomer or nucleic acids, which is preferably isotonic with the blood of the recipient. This preparation may be
25 formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation also may be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butane diol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium
30 chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be

employed including synthetic mono-or di-glycerides. In addition, fatty acids such as oleic acid may be used in the preparation of injectables.

Carrier formulation suitable for oral, subcutaneous, intravenous, intramuscular, etc. administrations can be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA.

In a preferred embodiment of the invention said pharmaceutical compositions modulates angiogenesis. Preferably said modulation is the inhibition of angiogenesis.

10 Preferably said inhibition relates to endothelial cell stimulated angiogenesis.

Alternatively, or preferably, said inhibition is the inhibition of macrophage and/or tumour cell stimulated angiogenesis.

15 In a further preferred embodiment of the invention said inhibition is mediated by the inhibition of pro-angiogenic factors. Ideally these are either intracellular or cell surface receptors.

Preferably, peptides/oligomers according to the invention can be manufactured by in vitro peptide synthesis using standard peptide synthesis techniques known to those skilled in the art, see for example, Merrifield B, Protein Science (1996), 5: 1947-1951; The chemical synthesis of proteins; Mutter M, Int J Pept Protein Res 1979 Mar;13(3):274-7 Studies on the coupling rates in liquid-phase peptide synthesis using competition experiments; and Solid Phase Peptide Synthesis in the series Methods in Enzymology (Fields, G.B. (1997) Solid-Phase Peptide Synthesis. Academic Press, San Diego. #9830).

Alternatively, polypeptides comprising the peptides of the invention can be manufactured by recombinant techniques which are well known in the art.

30

According to a further aspect of the invention there is provided a vector, wherein said vector includes a nucleic acid molecule which encodes for peptides and/or oligomers according to the invention.

- 5 Alternatively, vector(s) which include nucleic acid encoding said peptides/oligomers can be adapted for recombinant expression.

In a preferred embodiment of the invention said vector is an expression vector adapted for prokaryotic or eukaryotic cell expression. Preferably said eukaryotic
10 vector is adapted for gene therapy.

Typically said adaptation includes, by example and not by way of limitation, the provision of transcription control sequences (promoter sequences) which mediate cell/tissue specific expression. These promoter sequences may be cell/tissue specific,
15 inducible or constitutive.

Promoter is an art recognised term and, for the sake of clarity, includes the following features which are provided by example only, and not by way of limitation. Enhancer elements are cis acting nucleic acid sequences often found 5' to the transcription
20 initiation site of a gene (enhancers can also be found 3' to a gene sequence or even located in intronic sequences and is therefore position independent). Enhancers function to increase the rate of transcription of the gene to which the enhancer is linked. Enhancer activity is responsive to trans acting transcription factors (polypeptides) which have been shown to bind specifically to enhancer elements. The
25 binding/activity of transcription factors (please see Eukaryotic Transcription Factors, by David S Latchman, Academic Press Ltd, San Diego) is responsive to a number of environmental cues which include, by example and not by way of limitation, intermediary metabolites or environmental effectors.

30 Promoter elements also include so called TATA box and RNA polymerase initiation selection (RIS) sequences which function to select a site of transcription initiation.

These sequences also bind polypeptides which function, inter alia, to facilitate transcription initiation selection by RNA polymerase.

Adaptations also include the provision of selectable markers
5 and autonomous replication sequences which both facilitate the maintenance of said vector in either the eukaryotic cell or prokaryotic host. Vectors which are maintained autonomously are referred to as episomal vectors.

Adaptations which facilitate the expression of vector encoded genes include the
10 provision of transcription termination/polyadenylation sequences. This also includes the provision of internal ribosome entry sites (IRES) which function to maximise expression of vector encoded genes arranged in bicistronic or multi-cistronic expression cassettes.

15 These adaptations are well known in the art. There is a significant amount of published literature with respect to expression vector construction and recombinant DNA techniques in general. Please see, Sambrook et al (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory, Cold Spring Harbour, NY and references therein; Marston, F (1987) DNA Cloning Techniques: A Practical
20 Approach Vol III IRL Press, Oxford UK; DNA Cloning: F M Ausubel et al, Current Protocols in Molecular Biology, John Wiley & Sons, Inc.(1994).

In a yet further preferred embodiment of the invention there is provided a gene therapy vector comprising the nucleic acid according to the invention.

25 It will be apparent to one skilled in the art that the delivery of gene therapy vectors either to endothelial cells or tumour cells target the production of polypeptides according to the invention to the vicinity of the tumour thereby augmenting the anti-angiogenic effect of said polypeptides.

According to a yet further aspect of the invention there is provided a cell transformed/transfected with the nucleic acid according to the invention. Ideally said nucleic acid is the vector according to the invention.

- 5 According to yet still a further aspect of the invention there is provided a non-human, transgenic animal characterised in that said animal incorporates a nucleic acid molecule encoding a polypeptide according to the invention into its genome.

10 It will be apparent to one skilled in the art that the provision of non-human transgenic animals genetically modified by the provision of a transgene(s) encoding polypeptides according to the invention is an alternative source of active polypeptide. It is well known in the art that transgenic animals can be used to make various therapeutic polypeptides.

- 15 According to a further aspect of the invention there is provided a combined preparation comprising a peptide/oligomer according to the invention and at least one cytotoxic agent.

20 According to a yet further aspect of the invention there is provided a combined preparation comprising a peptide/oligomer according to the invention and at least one further anti-angiogenic agent.

25 Examples of cytotoxic agents are well known in the art, for example, and not by way of limitation cytotoxins, such as ricin A-chain or diphtheria toxin; antagonists of the key pro-angiogenic factors in tumours (eg VEGF, bFGF, TNF alpha, PDGF) would include neutralising antibodies or receptors for these factors, or tyrosine kinase inhibitors for their receptors (eg. SU5416 for the VEGF receptor, Flk-1/KDR); prodrug activating enzymes such as, human simplex virus-thymidine kinase HSV-TK, which activates the prodrug, ganciclovir when it is then administered systemically; chemotherapeutic agents, such as neocarzinostatin; cisplatin; 30 carboplatin; cyclophosphamide; melphalan; carmusline; methotrexate; 5-

fluorouracil; cytarabine; mercaptopurine; daunorubicin; doxorubicin; epirubicin; vinblastine; vincristine; dactinomycin; mitomycin C; taxol; L-asparaginase; G-CSF; an enediyne such as chaliceamicin or esperamicin; chlorambucil; ARA-C; vindesine; bleomycin; and etoposide.

5

Examples of anti-angiogenic agents are known in the art and include, by example and not by way of limitation, angiostatin and endostatin. Anti-angiogenic polypeptides and peptides are also disclosed in WO01/88129 and WO02/18440 respectively, which are incorporated by reference. Anti-angiogenic peptides are also disclosed in

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currently unpublished GB 0203882.6, which is incorporated by reference.

In a further aspect of the invention there is provided a method to treat an animal which would benefit from inhibition of cell-division comprising:

- 15 i) administering an effective amount of an agent comprising a peptide/oligomer according to the invention, to an animal to be treated;
- ii). monitoring the effects of said agent on the inhibition of cell-division.

20

In a preferred method of the invention said treatment is the inhibition of tumour development.

25

It will be apparent that the above combinations of peptides/oligomers and therapeutic agents will also have benefit with respect to the treatment of other conditions/diseases which are dependent on angiogenesis. For example, neovascular glaucoma, rheumatoid arthritis, psoriasis and diabetic retinopathy.

In a yet further alternative method of treatment, said agent is a gene therapy vector according to the invention.

In addition, said nucleic acid encoding a peptide/oligomer according to the invention is provided with, nucleic acid encoding an agent which augments the anti-angiogenic effect of said peptide/oligomer.

- 5 According to a yet further aspect of the invention there is provided an imaging agent comprising a peptide/oligomer according to the invention.

It will be apparent to the skilled artisan that peptides according to the invention can be used to target imaging agents to, for example, tumours, to identify developing
10 tumours or to monitor the effects of treatments to inhibit tumour growth. It will also be apparent that the combined therapeutic compositions which comprise both peptides and a further anti-angiogenic agent may be further associated with an imaging agent to monitor the distribution of the combined therapeutic composition and/or to monitor the efficacy of said combined composition.

15

Methods used to detect imaging agents are well known in the art and include, by example and not by way of limitation, positron emission tomographic detection of F^{18} and C^{11} compounds.

- 20 An embodiment of the invention will now be described by example only and with reference to the following figures:

Figure 1 illustrates the anti-angiogenic effect of peptide Y after 6hrs incubation;

- 25 Figure 2 illustrates the cytotoxic effect of peptide Y on *in vitro* cultures of HuDMEC's;

Figure 3 illustrates the apoptotic effect of peptide Y on *in vitro* cultures of HuDMEC's;

- 30 Figure 4 illustrates the cell-cycle inhibitory effect of peptide Y on *in vitro* cultures of HuDMEC's;

Figure 5 illustrates the effect of pre-incubation of a $\alpha v\beta 3$ antibody on tubule formation of *in vitro* cultures of HuDMEC's; and

5 Figure 6 is the (a) DNA and (b) amino acid sequence of peptide Y.

EXAMPLE 1

Effects of 6 hours exposure to NpY(18-36)

10

Experiment A: Matrigel

1. Spread 30 μ l Matrigel per well and allow to set at room temperature for 15 minutes
2. Lift HuDMECs by trypsinisation, spin down and resuspend in DMEM + 1%FCS
- 15 3. Seed HuDMECs into Matrigel covered wells (20,000 cells/well) in the presence or absence of VEGF (10ng/ml) with or without 1 μ M NpY(18-36).
4. Allow the plate to stand in the hood without moving for 30 minutes, to enable to cells to attach without ending up round the edges of the wells.
5. Transfer the plate to the incubator (37°C) for 6 hours
- 20 6. Fix the plate with ce-cold 70% ethanol
7. Rinse in water and stain using haematoxylin and eosin, then air dry the plate
8. Analyse two fields of view per well using Scion Image.

Results are illustrated in Figure 1.

25

30

EXAMPLE 2**Cytotoxicity**

1. Lift cells by trypsinisation and seed into 24 well plates (100,000 cells/well) in the presence or absence of VEGF and nothing or 1 μ M NpY (18-36).
2. Incubate for 6 hours at 37°C.
3. Remove and keep the media in labelled eppendorfs (containing floating, dead cells) and wash the wells with PBS, again adding this to the eppendorfs and centrifuge the eppendorfs for 5 minutes.
4. Lift the remaining cells with trypsin and add to the relevant eppendorfs and spin down.
5. Pour off the supernatant and resuspend the cells in 200 μ l and transfer to FACs tubes.
6. Add 25 μ l high strength propidium iodide to each tube and read on FACS.

Results are illustrated in Figure 2.

EXAMPLE 3**Annexin V staining for apoptosis.**

1. HUDMECs were lifted by trypsinisation and seeded at 100,000 cells/well in DMEM + 1%FCS over agarose with or without VEGF (10ng/ml) in the presence or absence of 1 μ M NpY(18-36).
2. The cells were incubated for 6 hours at 37°C.
3. After 6 hours the cells were spun down and washed by resuspending in PBS and centrifuging again. This was repeated twice.
4. After the second wash the supernatant was poured off and the cells resuspended in 100 μ l Annexin V buffer. 2.5 μ l Annexin V solution and 10 μ l propidium iodide solution was added per treatment and the cells incubated at room temperature in the dark for 15 minutes,

5. After this time a further 300µl Annexin V buffer was added to each tube and the cells transferred to FACs tubes for analysis on the FACs within an hour.

Results are illustrated in Figure 3.

5

EXAMPLE 4

Cell cycle analysis

1. Cells were lifted by trypsinisation and plated into 24 well plates at 150,000 cells/well.
2. The cells were treated with nothing (control) or 1µM NpY(18-36) and incubated for 6 hours.
3. After 6 hours the cells were lifted by trypsinisation (including floating cells) and washed twice in PBS.
- 15 4. The cells were fixed in ice-cold 70% ethanol at -20°C overnight.
5. The cells were centrifuged for 2 minutes, the ethanol supernatant removed and the cells resuspended and washed twice in PBS.
6. The cell pellets were then resuspended in 300µl PBS containing 1mg/ml PI and 10 units/ml RNase at room temperature in the dark for a minimum of 2 hours.
- 20 7. The samples were then run on the FACs.

Results are illustrated in Figure 4.

EXAMPLE 5

25

Matrigel using $\alpha_v\beta_3$ blocking antibody.

1. Spread 30µl Matrigel per well and allow to set at room temperature for 15 minutes
- 30 2. Lift HuDMECs by trypsinisation, spin down and resuspend in DMEM + 1%FCS

3. Seed HuDMECs into Matrigel covered wells (20,000 cells/well) and allow the cells to attach without moving for 30 minutes.
4. After this time either an antibody to $\alpha_v\beta_3$, 23C6 (10 μ g/ml), or a negative control antibody (10 μ g/ml) is added to the medium for a further 30 minutes.
5. At this stage 1 μ M NpY(18-36) was added to the wells in the presence of 10ng/ml VEGF and the plate is transferred to the incubator (37°C) overnight.
6. Fix the plate with ice-cold 70% ethanol
7. Rinse in water and stain using haematoxylin and eosin, then air dry the plate
8. Analyse three fields of view per well using Scion Image.

10

Results are illustrated in Figure 5

In vivo Animal Model Studies

- 15 Experiments were performed on six-week-old Balb/C mice weighing 15g, obtained from Sheffield Field Laboratories. All experiments were approved by the Home Office Project Licence Number PPL50/1414.

Tumour Cell Culture

20

The CT26 cell line was maintained by *in vitro* passage in Dulbecco's Minimal Eagles Medium containing 10% foetal calf serum, and 1% penicillin and streptomycin and maintained at 37°C in humidified atmosphere of 5% CO₂ in air. The cell line was routinely checked to ensure freedom from mycoplasma (Mycoplasma rapid detection system, Gena-Probe Incorporated, U.S.A.).

25

Subcutaneous Tumour Implantation

- 30 Animals were anaesthetised with an intraperitoneal injection of diazepam (0.5mg/ml, Dumex Ltd.) and hypnorm (fentanyl citrate 0.0315mg/ml and fluanisone 1mg/ml, Janssen Pharmaceutical Ltd.) in the ratio of 1:1 at a volume of 0.1ml/200g body weight, with supplementation as required to maintain adequate anaesthesia. Naïve

Balb/c mice were immunised s.c into the right flank, following removal of the fur. Tumour cells were injected at a concentration of 3×10^5 viable CT26 cells per animal suspended in 100ul serum free medium. Animals were then allowed to recover. Tumour growth and animal weights were monitored daily.

5

Administration of Peptides

Tumour growth was measured daily and when the majority of animals in the cohort had tumour volumes of $>100\text{mm}^3$ but $<350\text{mm}^3$ animals were divided into experimental and control groups. This occurred between 14 and 18 days following implantation of the tumour cell suspension. Animals then received an intraperitoneal (ip) injection of either active drug (peptide at 100mM; 100ul) or vehicle (phosphate buffered saline, 100ul). Daily injections continued until the tumour growth in the control animals reached the maximum burden allowed by Home Office legislation.

15

Assessment of tumour growth

Tumour volumes were assessed by calliper measurements of the perpendicular diameters and volumes estimated using the equation:-

20

$$\text{Volume} = (a^2 \times b)/2$$

where a is the smaller and b the larger diameter.

Animals were weighed on a daily basis and the general well being monitored.

25

Statistical Analysis.

All experiments were performed at least three times and data analysed using the Mann-Whitney U test, a non-parametric test that does not assume a Gaussian distribution in the data being analysed. $P \leq 0.05$ was taken as significant.

30

Claims

1. An isolated nucleic acid molecule which encodes a peptide characterised in
5 that the nucleic acid molecule is selected from the following group:
 - i) a nucleic acid molecule comprising the nucleic acid sequence presented in figure 6;
 - ii) a nucleic acid molecule as represented by the sequence presented in figure 6 which has been modified by addition, deletion or substitution of at least one
10 nucleotide base within at least one codon to encode a variant peptide which has cell-cycle inhibitory activity;
 - iii) a nucleic acid molecule which hybridizes to the sequence in (i) or (ii); and
 - iv) a nucleic acid molecule comprising a nucleic acid sequence which is degenerate as a result of the genetic code to the sequences identified in (i)-
15 (iii); for the manufacture of a medicament for use in the treatment of diseases or conditions which would benefit from an inhibition of cell-division.
2. A peptide encoded by the nucleic acid according to Claim 1 for the manufacture of a medicament for use in the treatment of diseases or conditions which
20 would benefit from the inhibition of cell division.
3. A peptide according to Claim 2 wherein said peptide inhibits angiogenesis.
4. A peptide according to Claim 2 or 3 wherein said disease is selected from the
25 group consisting of: cancer; psoriasis; neovascular glaucoma; rheumatoid arthritis; diabetic retinopathy.
5. A peptide according to Claim 4 wherein said disease is cancer.
- 30 6. A peptide according to Claim 2 or 3 wherein said disease is psoriasis.

7. A peptide according to Claim 6 wherein said psoriatic condition is selected from the group consisting of: nail psoriasis; scalp psoriasis; plaque psoriasis; pustular psoriasis; guttate psoriasis; inverse psoriasis; erythrodermic psoriasis; psoriatic arthritis.

5

8. A peptide according to any of Claims 2-7 wherein said peptide comprises an amino acid sequence, or part thereof, consisting of the amino acid sequence ARYYALSRLHYINLITRQRT.

10 9. A peptide according to Claim 8 wherein said peptide is a peptide consisting of the amino acid sequence ARYYALSRLHYINLITRQRT.

10. A peptide according to any of Claims 2-9 wherein said peptide is a fragment of the peptide ARYYALSRLHYINLITRQRT.

15

11. A peptide according to any of Claims 2-10 wherein said peptide is acetylated.

12. A peptide according to Claim 11 wherein said acetylation is to the amino terminus of said peptide.

20

13. A peptide according to any of Claims 2-12 wherein said peptide is amidated.

14. A peptide according to Claim 13 wherein said amidation is to the carboxyl-terminus of said peptide.

25

15. A peptide according to any of Claims 2-10 wherein said peptide, or fragment thereof, is modified by both acetylation and amidation.

16. A peptide according to any of Claims 2-15 wherein said peptide is modified by cyclisation.

30

17. An agent comprising two or more peptides according to any of Claims 2-16 wherein said agent has cell-cycle inhibitory activity.

18. An agent according to Claim 17 wherein said two or more peptides are linked
5 by a linker molecule.

19. An agent according to Claim 17 or 18 wherein said agent comprises a plurality of peptides.

10 20. An agent according to Claim 19 wherein said agent comprises 3, 4, 5, 6, 7, 8, 9, or 10 peptides linked together as an oligomeric peptide.

21. An agent according to Claim 17 or 18 wherein said peptide has greater than
15 10 peptides.

22. An agent according to Claim 17 or 18 wherein said agent is a dimer of two peptides.

23. An agent according to any of Claims 17-22 wherein said linker is a peptide
20 linking molecule.

24. An agent according to Claim 23 wherein said peptide linking molecule comprises at least one amino acid residue which links at least two peptides.

25 25. An agent according to Claim 23 or 24 wherein said peptide linking molecule comprises at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acid residues.

26. An agent according to Claim 23 wherein said linking molecule comprises
30 more than 10 amino acid residues.

27. An agent according to any of Claims 17-26 wherein said agent is a fusion protein comprising an inframe translational fusion.

28. The use of an agent according to any of Claims 17-27 as a pharmaceutical.

5

29. A pharmaceutical composition comprising an agent according to any of Claims 17-27.

10 30. A vector comprising a nucleic acid molecule which encodes a peptide and/or agent according to any of Claims 2-27.

31. A cell transformed/transfected with a nucleic acid molecule according to Claim 1 or a vector according to Claim 30.

15 32. A non-human, transgenic animal characterised in that said animal incorporates a nucleic acid molecule encoding a peptide and/or agent according to any of Claims 2-27.

20 33. A combined preparation comprising a peptide/agent according to any of Claims 2-27 and at least one cytotoxic agent.

34. A combined preparation comprising a peptide/agent according to any of Claims 2-27 and at least one anti-angiogenic agent.

25 35. A method to treat an animal which would benefit from inhibition of cell-division comprising:

- i) administering an effective amount of an agent comprising a peptide/agent according to any of Claims 2-27, to an animal to be treated;
- ii). monitoring the effects of said agent on the inhibition of cell-division.

30

36. A method according to Claim 35 wherein said treatment is the inhibition of tumour development.

37. A method according to Claim 35 or 36 wherein said agent is a nucleic acid molecule according to Claim 1 or a vector according to Claim 30.

38. An imaging agent comprising a peptide/agent according to any of Claims 2-27.

39. A peptide comprising the amino acid sequence ARYYALSRLHYINLIRQRT, or a variant peptide wherein said sequence is modified by addition, deletion or substitution of at least one amino acid residue, for use as a pharmaceutical agent.

40. A pharmaceutical composition comprising a peptide according to Claim 39.

Figure 1

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* $P < 0.02$ with respect to relevant control group

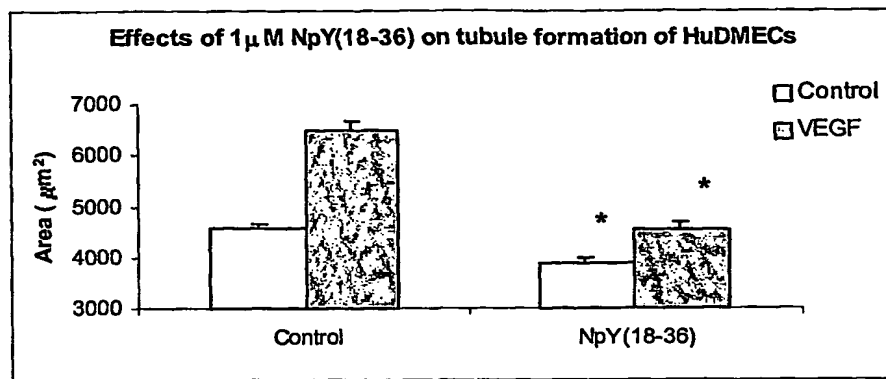


Figure 2

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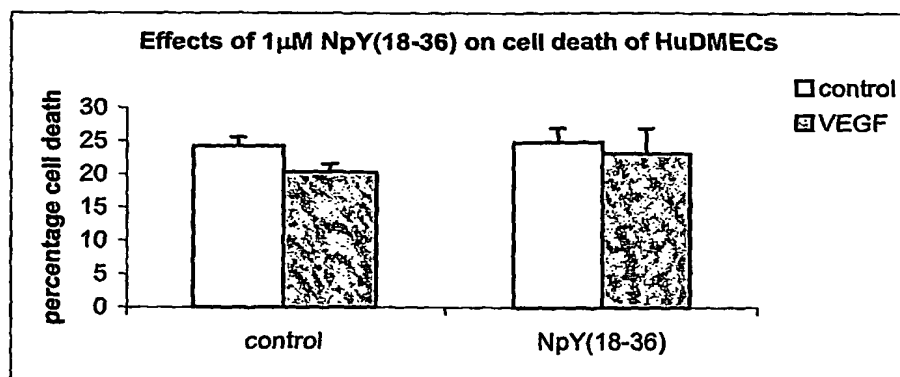


Figure 3

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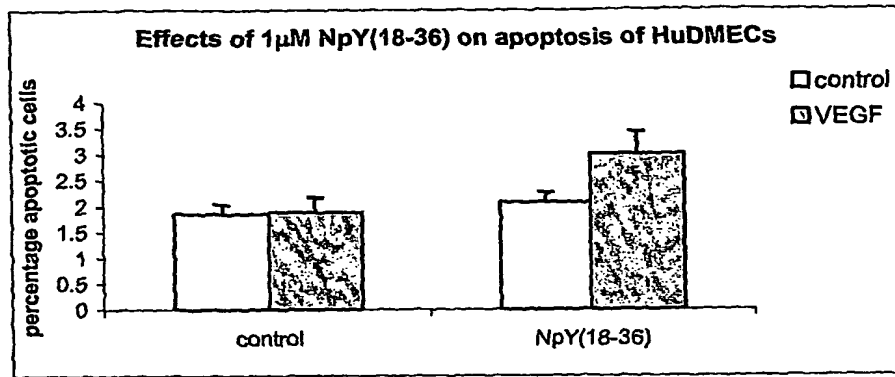


Figure 4

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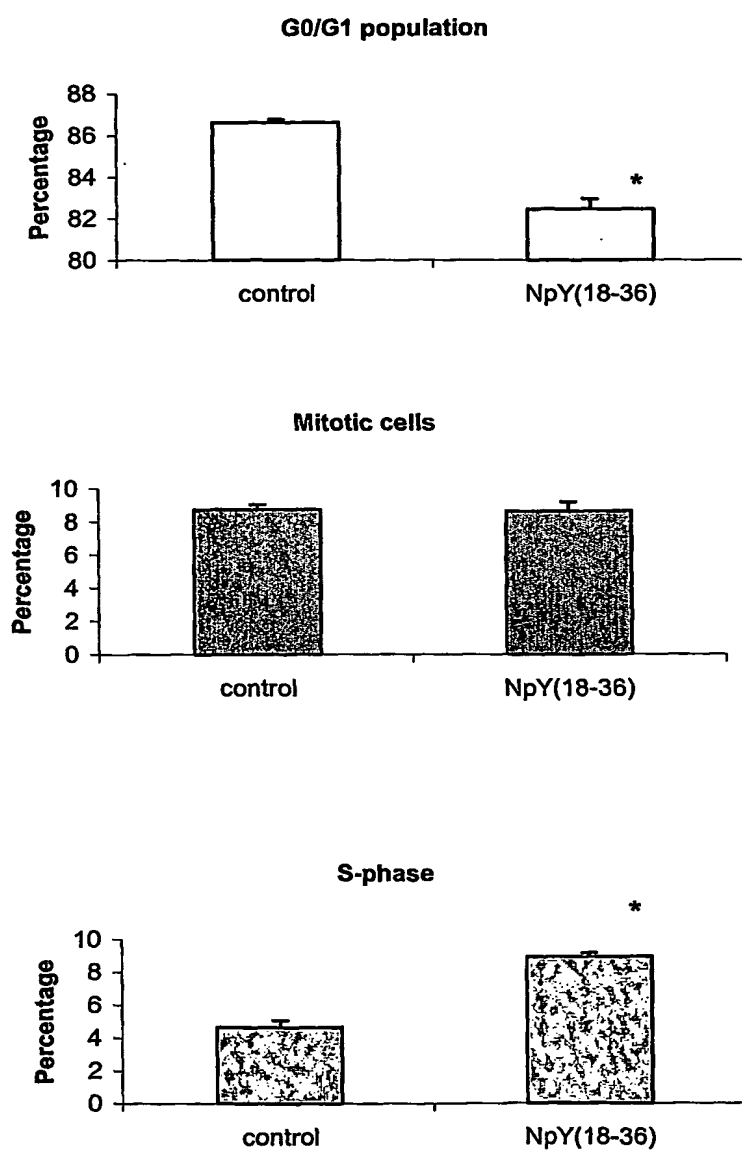


Figure 5

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*P<0.005 with respect to relevant control group.

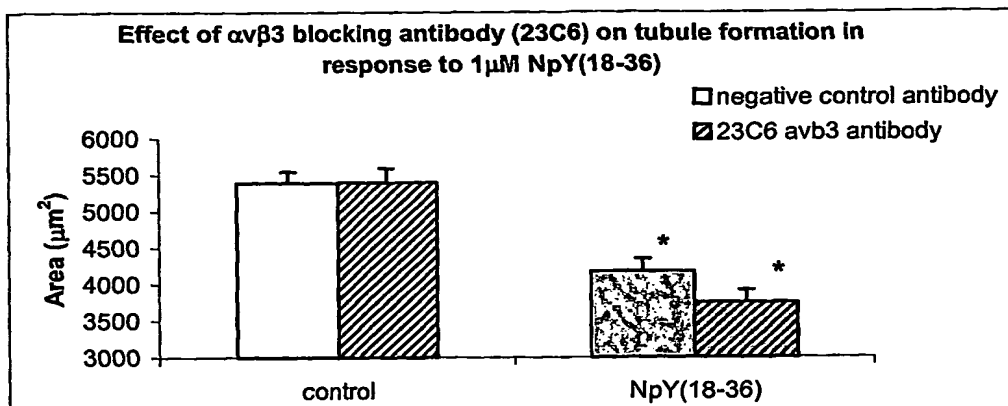


Figure 6

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a

GCA AGA TAC TAT AGT GCA TTA AGA CAT TAT ATA AAT TTA ATA ACA AGA CAA AGA ACA

b

ARYYSALRHYNLITRQRT

INTERNATIONAL SEARCH REPORT

International Application No
PCT 03/23140

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07C217/18 C07C217/14 A61K31/135

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07C A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95 04277 A (SPHINX PHARMA CORP) 9 February 1995 (1995-02-09) page 9, last paragraph; page 44, scheme VI	1, 33
A	EP 0 551 662 A (BAYER AG) 21 July 1993 (1993-07-21) abstract; claims 1, 7, 8	1, 33
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-/-		

☒ Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search

11 December 2003

Date of mailing of the international search report

23/12/2003

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INTERNATIONAL SEARCH REPORT

International Application No

PC 03/03089

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K14/575 A61K38/22 C12N15/10 A01K67/027 C12N15/86
G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	whole document, abstract, lines 3/4, esp. page 4640	1-41
Y	WO 95 00161 A (UNIV CINCINNATI ;BALASUBRAMANIAM AMBIKAIPAKAN (US)) 5 January 1995 (1995-01-05) Calim 12 and related examples, Table II, page 42 ff, Example 5 --- -/--	1-41

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Name and mailing address of the ISA

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INTERNATIONAL SEARCH REPORT

International Application No

PC 03/03089

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Y	YU A ET AL: "Vitamin E and the Y4 agonist BA-129 decrease prostate cancer growth and production of vascular endothelial growth factor" JOURNAL OF SURGICAL RESEARCH, vol. 105, no. 1, 1 June 2002 (2002-06-01), pages 65-68, XP002262057 ISSN: 0022-4804 Gastriintestinal hormaone peptide, similar to neuropeptide y	1-41
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INTERNATIONAL SEARCH REPORT

International Application No

PC 03/03089

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PC 03/03089

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